

## BASIC SCIENCE: OBSTETRICS

# Fetal responses to lipopolysaccharide-induced chorioamnionitis alter immune and airway responses in 7-week-old sheep

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**OBJECTIVE:** We hypothesized that fetal innate immune responses to lipopolysaccharide-induced chorioamnionitis would alter postnatal systemic immune and airway responsiveness.

**STUDY DESIGN:** Ewes received intraamniotic injections with saline or lipopolysaccharide at 90, 100, and 110 days of gestation. Immune status and airway responsiveness were evaluated at term and at 7 weeks of age.

**RESULTS:** At term, lymphocytes, monocytes, and neutrophils were significantly increased (respectively, 24-fold, 127-fold, and 31,000-fold) in lungs and blood monocytes became Toll-like receptor 2 responsive

after lipopolysaccharide exposures. Furthermore, CD4 and CD4/CD25 lymphocytes were increased in thymus and lymph nodes. At 7 weeks, airway reactivity decreased and concentrations of CD8 cytotoxic T lymphocytes changed in the lungs and thymus relative to controls.

**CONCLUSION:** Early gestational lipopolysaccharide exposure increased leukocyte responsiveness at term. Decreased airway reactivity and changes in lymphocytes at 7 weeks postnatal demonstrate persistent effects of fetal exposure to LPS.

**Key words:** airway reactivity, hygiene hypothesis, inflammation, preterm

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Preterm birth (<37 weeks) is frequently associated with chorioamnionitis.<sup>1</sup> Recently, chorioamnionitis was correlated with a 4-fold increased risk for developing asthma in preterm infants after correction for confounding factors.<sup>2-4</sup> This observation is in contrast to the hygiene hypothesis,<sup>5-7</sup> in which infections during early childhood are associated with a decreased risk of subsequent asthma and allergic disease. These conflicting observations suggest that the

time of exposure to bacterial products during childhood may have different effects when compared with antenatal exposures. For example, antenatal exposure to bacterial products may increase the risk of asthma in childhood, whereas postnatal exposure to bacterial products may protect from asthma. The mechanisms to explain the different outcomes are still unclear.

Chorioamnionitis-mediated inflammation exposes the fetus to proinflam-

matory mediators primarily by fetal breathing and swallowing of amniotic fluid.<sup>8-10</sup> The fetal lung responds to mediators such as lipopolysaccharide (LPS), interleukin-1 (IL-1), and live ureaplasma with a local inflammatory response. Previous experiments revealed that exposure to chorioamnionitis changes the lymphocyte population in the posterior mediastinal lymph node (PMLN) that drains the lung<sup>11,12</sup> and causes systemic immune modulation of blood monocytes.<sup>13-15</sup> Furthermore, the gut responses to LPS with altered inflammatory cells in the gut wall and regional lymph nodes.<sup>8,16</sup> These acute fetal responses were modulated further with repeated exposure to induce LPS-tolerance and cross-tolerance to other proinflammatory Toll-like receptor (TLR) agonists.<sup>13-15</sup> However, the relationship between exposure to antenatal inflammation and postnatal disease is confounded by gestational age and neonatal lung disease in human studies.

We hypothesized that fetal innate immune responses to LPS-induced chorioamnionitis would alter postnatal sys-

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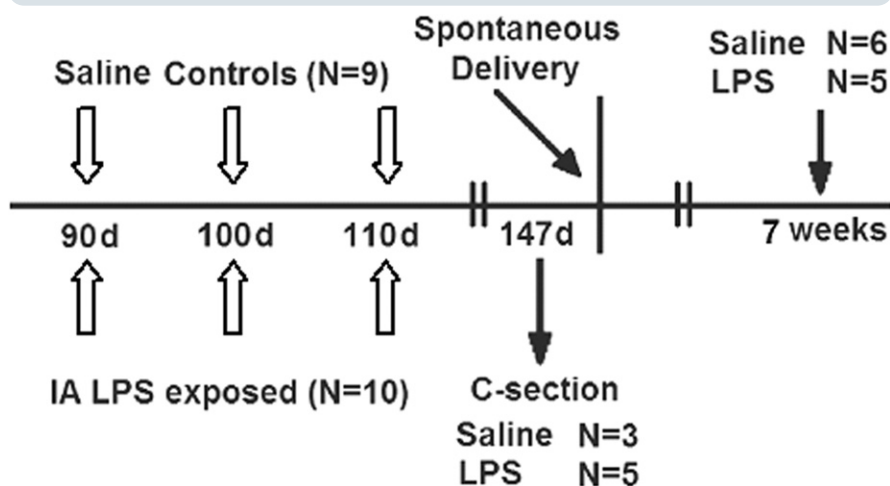
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**FIGURE 1**  
**Timing of fetal exposure to LPS**



Open arrows indicate gestational age of intraamniotic (IA) injections with LPS or saline. Sheep model with LPS-induced chorioamnionitis. Operative delivery was performed at 147 days gestation for some animals ( $n = 3$ -5 lambs), whereas the remaining ewes ( $n = 5$ -6) delivered spontaneously and their lambs were studied at 7 weeks. Immune status was evaluated in postmortem tissues at 147 days and at 7 weeks.

LPS, lipopolysaccharide.

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temic immune and airway responsiveness resembling asthma later in life. To test this hypothesis, we evaluated immune status at birth and at 7 weeks after LPS-mediated chorioamnionitis. We evaluated leukocytes in the bronchoalveolar lavage fluid (BALF). Transforming growth factor-beta 1 ( $\text{TGF-}\beta_1$ ) and elastin were measured in lung tissue. CD4, CD8, CD4/CD25, and gamma-delta subpopulations of T lymphocytes were measured in the PMLN and thymus. Monocytes isolated from blood were tested in vitro for responses to LPS and PamCysK4 (a ligand for TLRs 1 and 2). The immunologic measurements were performed at term and at 7 weeks of age and airway reactivity to metacholine (MCh) was tested at 7 weeks after inhalation of house dust mite (HDM).

## MATERIALS AND METHODS

### Antenatal treatment

All animal procedures were approved by the Animal Ethics Committee of the University of Western Australia, Australia. Ewes received intraamniotic (IA) injections with saline (controls) or 10 mg

*Escherichia coli* 055:B5 endotoxin (LPS; Sigma-Aldrich, St. Louis, MO) at 90, 100, and 110 days of gestation (Figure 1). At 147 days of gestation, lambs from LPS ( $n = 5$ ) and control ( $n = 3$ ) groups were delivered surgically to assess immune function of the fetus at term (term is approximately 147 days). The remaining ewes delivered spontaneously and airway responsiveness and immune status of lambs were evaluated at 7 weeks (LPS  $n = 5$ , Control  $n = 6$ ). Lambs were killed with intravenous pentobarbital at term and at 7 weeks.

### Airway reactivity

At 7 weeks lambs were sedated 2 days after HDM exposure (nebulized 1 mg) with diazepam 0.25 mg/kg (Sigma NSW, Australia) and Ketamine 5 mg/kg (Parnell Laboratories, NSW, Australia). They were intubated with a 6.0 mm ID tracheal tube (Portex Ltd, UK) and ventilated (Humming V, Metran, Japan) with a peak inspiratory pressure of 25 cm  $\text{H}_2\text{O}$ , an inspiratory time 0.7 second and a rate of 40 breaths per minutes. A continuous infusion of intravenous Propofol (0.3-0.6 mg/kg/h, Repose, 0.1 mg/kg/

min; Norbrook Laboratories Ltd., Victoria, Australia) and Remifentanyl (0.3 mg/kg/h, Ultiva 0.05  $\mu\text{g/kg/min}$ ; Glaxo-SmithKline, Victoria, Australia) was commenced, and following confirmation of deep anesthesia, neuromuscular blockade was achieved with vecuronium (0.1 mg/kg IV; Essex Pharma, Germany). Lambs were stabilized for 10 minutes before baseline measurements, and then received for 1 minute aerosols (1 mL) at 5 minute intervals of saline, followed by increasing concentrations (0.01%, 0.03%, 0.1%, 0.3%, 1%; w/v) of methacholine (Acetyl- $\beta$ -methylcholine chloride; Sigma). Partitioned respiratory impedance ( $Z_{rs}$ ) was measured using the low-frequency forced oscillation technique (LFOT) using the FlexiVent (Module 5; Scireq, Montreal, Canada). After airway occlusion at commencement of expiration, respiratory system input impedance ( $Z_{rs}$ ) measurements were obtained using a primewave (17 mutually prime frequencies from 0.5-19.75 Hz) over a 6-second apneic interval. Measurements were repeated every 30 seconds until peak response was observed (normally approximately 3 minutes).

Measurements of pressure and flow were transformed to the frequency domain and corrected for the impedance of the tracheal tube and measurement system. The partitioned airway (airway resistance -  $R_{aw}$ ; airway inertance -  $I_{aw}$ ) and tissue mechanical variables (tissue damping -  $G$ ; tissue elastance -  $H$ ) were determined by fitting the resultant impedance spectrum to the constant phase model.<sup>17</sup> Hysteresivity ( $\eta$ ) was calculated as  $G/H$ . Changes in airway and tissue variables were expressed relative to measurements obtained at baseline.

### Bronchoalveolar lavage

After collection of the lungs, thymus, and the PMLN, the left lung was lavaged 3 times with 0.9% NaCl.<sup>18</sup> The BALFs were pooled and centrifuged at 500 rpm for 5 minutes. Differential cell counts were performed on cytospin preparations after staining with Pappenheim-staining (May-Grünwald, Giemsa).<sup>18</sup>

**TABLE 1**  
**Physiology**

| Antenatal treatment | Body weight, kg | PMLN, g/kg  | Thymus, g/kg | Spleen, g/kg | Total lung, g/kg | Animals, n |
|---------------------|-----------------|-------------|--------------|--------------|------------------|------------|
| At term             |                 |             |              |              |                  |            |
| Control             | 5.0 ± 1.0       | 0.13 ± 0.01 | 2.07 ± 1.07  | 1.37 ± 0.18  | 35.3 ± 3.3       | 3          |
| LPS                 | 5.5 ± 1.0       | 0.21 ± 0.02 | 3.59 ± 0.43  | 1.74 ± 0.20  | 31.8 ± 2.4       | 5          |
| 7 weeks             |                 |             |              |              |                  |            |
| Control             | 16.2 ± 1.3      | 0.23 ± 0.02 | 2.52 ± 0.19  | 4.20 ± 0.30  | 12.9 ± 0.4       | 6          |
| LPS                 | 15.5 ± 1.5      | 0.26 ± 0.02 | 2.68 ± 0.48  | 4.62 ± 0.50  | 13.7 ± 0.4       | 5          |

Body weight, organ weights, and number of animals studied at birth and at 7 weeks of age. Organ weights are standardized per kilogram body weight. There were no differences to corresponding control group.

LPS, lipopolysaccharide; PMLN, posterior mediastinal lymph node.

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## Lung tissue

Lung tissue from the right lower lobe (RLL) was snap frozen and stored at  $-80^{\circ}\text{C}$ . For homogenization, a mix of lysis buffer (RIPA buffer, Sigma) and protease inhibitor (Sigma) was added to lung tissue. The lung tissue was homogenized (PRO Quick Connect Generators part no. 02-07095; PRO Scientific Inc., Oxford, CT) and centrifuged at 12 rcf (11.4 rpm) for 5 minutes at  $4^{\circ}\text{C}$ .

## Enzyme-linked immunosorbent assay of TGF- $\beta_1$

Free, bound, and total TGF- $\beta_1$  (referred to R&D enzyme-linked immunosorbent assay [ELISA] kit; Minneapolis, MN as active, latent, and total TGF- $\beta_1$ ) were measured with R&D ELISA kits (DuoSet ELISA, human TGF- $\beta_1$ , no. DY240; R&D Systems). Free TGF- $\beta_1$  was measured for the original sample. Total TGF- $\beta_1$  was measured after acid activation of 150  $\mu\text{L}$  of the sample with 30  $\mu\text{L}$  1 M HCl for 10 minutes at room temperature and 33  $\mu\text{L}$  1 M NaOH HEPES was used to stop the activation.<sup>19</sup> Bound TGF- $\beta_1$  was calculated as the difference between total TGF- $\beta$  and free TGF- $\beta_1$ .

## Elastin staining

We measured elastin foci of secondary crests (concentrated vs nonconcentrated) and elastin fiber deposition in the vessel walls. Elastin fibers were stained black with a Hart's resorcin fuchsin solution,<sup>20-22</sup> using paraffin slides that were dehydrated and stained at  $60^{\circ}\text{C}$  and counterstained with a tartrazine solution. A magnification of  $20\times$  was used to

save 3 pictures randomly. Counting of elastin foci was blinded, using ImageJ 1.41o software (Rasband; National Institutes of Health, Bethesda, MD) for quantification.

## Flow cytometry

Single cell suspensions of thymus and PMLN were made with a strainer, assessed for viability, and counted using a Neubauer chamber (Hawksley, England).<sup>23</sup> The  $10^6$  cells were incubated with primary monoclonal antibodies (mAbs: CD4, CD8, CD25, and TcR-1 receptor [gamma-delta T lymphocytes]; VMRD USA) for 30 minutes at  $4^{\circ}\text{C}$ . Cells were washed and incubated with fluorescence-conjugated secondary mAbs for 30 minutes at  $4^{\circ}\text{C}$  in the dark (FITC and R-PE labeled antibodies from SEROTEC, Great Britain). Cells were analysed on a FACScalibur instrument (Becton Dickinson, Franklin Lakes, NJ) using CellQuest software (Becton Dickinson). CD4/CD25 percentages are expressed relative to the population of cells expressing CD4. Other lymphocyte percentages are expressed relative to the total population of thymic lymphocytes.<sup>11</sup>

## Hydrogen peroxide assay for blood monocytes

Monocytes and macrophages were isolated with Percoll gradients from cord blood (at term) and peripheral blood (at 7 weeks) to perform functional tests.<sup>15</sup> In brief, the isolated cells were cultured in RPMI 1640 media supplemented by 10% heat-inactivated fetal calf serum for 6 hours with LPS (a ligand for TLR 4; 100

ng/mL; *E coli* O55:B5; Sigma-Aldrich) and PamCysK4 (a synthetic ligand for TLRs 1 and 2; 5  $\mu\text{g/mL}$ ; EMC Microcollection, Tuebingen, Germany). PamCysK4 signals through the TLR2 pathway and is involved in maturation of the immune system. Control cells were exposed to saline (media). After washing the cells with phosphate buffered saline (PBS), the production of hydrogen peroxide by  $1 \times 10^6$  monocytes was measured with an assay based on the oxidation of ferrous iron ( $\text{Fe}^{2+}$ ) to ferric iron ( $\text{Fe}^{3+}$ ) by hydrogen peroxide under acidic conditions (Bioxytech  $\text{H}_2\text{O}_2$ -560 assay; OXIS International, Portland, OR).<sup>13,15</sup>

## Statistical analysis

Microsoft Excel and GraphPad Prism 5 were used for statistics. Data are shown as mean  $\pm$  SEM. Statistical differences between LPS and control groups were evaluated with Student *t* test and the Mann-Whitney test. Values were considered significant if  $P \leq .05$  vs the control (saline) group.

## RESULTS

### Body and organ weights at birth and at 7 weeks of age

The body weights of LPS-exposed lambs were similar to the control group at term and at 7 weeks (Table 1) indicating similar growth and development. The organ weights (PMLN, thymus, spleen, total lung) were not different for the groups at birth or at 7 weeks of age.

**TABLE 2**  
**Ventilatory variables of lambs during methacholine challenge**

| Treatment     | PaO <sub>2</sub> , mm Hg | PaCO <sub>2</sub> , mm Hg | RR, breaths/min | PIP, cm H <sub>2</sub> O | HR, bpm  |
|---------------|--------------------------|---------------------------|-----------------|--------------------------|----------|
| Saline (7 wk) | 96 ± 4                   | 53 ± 4                    | 26.5 ± 0.5      | 25.8 ± 1.9               | 175 ± 12 |
| LPS (7 wk)    | 98 ± 1                   | 51 ± 1                    | 25.1 ± 0.7      | 26.8 ± 2.3               | 161 ± 7  |

HR, heart rate; LPS, lipopolysaccharide; PaCO<sub>2</sub>, arterial carbon dioxide tension; PaO<sub>2</sub>, arterial oxide tension; PIP, peak-inflation pressure; RR, respiratory rate.

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### Airway reactivity

Measurements with methacholine challenge at 7 weeks, including heart rate, peak inspiratory pressure, respiratory rate, and blood gas values (PaCO<sub>2</sub>, PaO<sub>2</sub>) were not different between fetal LPS exposed and control lambs (Table 2). Representative impedance spectra are shown in Figure 2. There were no differ-

ences in baseline measurements between the groups. Airway resistance ( $R_{aw}$ ;  $P = .008$ ; Figure 2, A) and tissue damping ( $G$ ;  $P = .037$ ; Figure 2, B) did not increase at 1% w/v MCh in LPS-exposed lambs, whereas they increased in the saline control animals, indicating decreased airway reactivity with fetal LPS exposure. LPS-exposed lambs and control lambs did not

respond to lower MCh dosing. Tissue elastance ( $H$ ; Figure 2, C) and hysteresivity ( $\eta$ ; Figure 2, D) were not different at any MCh concentration between LPS-exposed and control lambs.

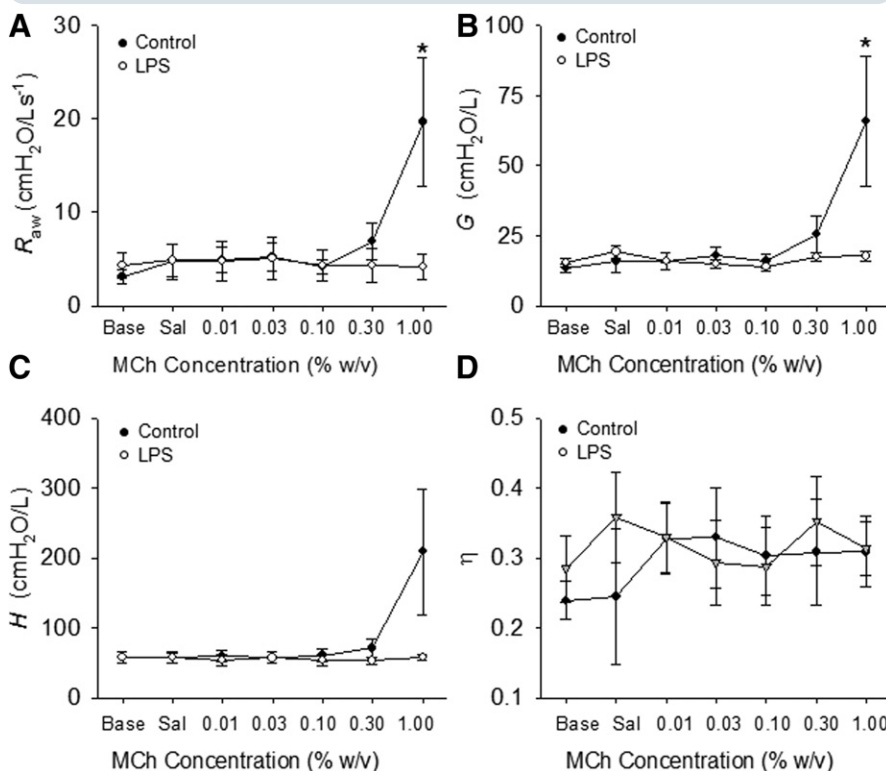
### Pulmonary inflammation

Monocytes, lymphocytes, and neutrophils were greatly increased in the BALF from the LPS-exposed term fetal lambs relative to the controls 37 days after the last IA injection ( $P < .001$  vs control, Figure 3, A). However, by 7 weeks there were no significant differences in the number of inflammatory cells in BALF for the LPS-exposed and control lambs (Figure 3, B).

### Hydrogen peroxide responses of blood monocytes

After stimulation in vitro with LPS, blood monocytes from control term lambs had no increased response, but after PamCysK4 stimulation a 6-fold increase was present (Figure 4, A). At 7 weeks, blood monocytes from control lambs responded to LPS and PamCysK4 (16-fold and 14-fold increases respectively; Figure 4, B). This change of monocyte responses between term and 7 weeks is explained by the maturation of the innate immune system in early post-natal life. LPS-exposed animals had a different monocyte response pattern. At term a stimulation in vitro with LPS and PamCysK4 increased H<sub>2</sub>O<sub>2</sub> 5-fold and 9-fold, respectively (Figure 4, A). This increase was still present at 7 weeks: 6-fold increase with LPS and 5-fold increase with PamCysK4 (Figure 4, B). Comparing term LPS-exposed lambs and control lambs after a specific stimulation, an increased monocyte response was only present after stimulation with PamCysK4 (Figure 4, A). At 7 weeks, no differences in the response were evident

**FIGURE 2**  
**Dose response curves to inhaled methacholine**



Responses to inhaled methacholine are expressed relative to measurements at baseline for 7-week old lambs that were exposed to intraamniotic injections of LPS or saline (control). A, Airway resistance, B, tissue damping, C, tissue elastance, and D, hysteresivity were measured to test the airway reactivity. Airway resistance ( $R_{aw}$ ;  $P = .008$ ) and tissue damping ( $G$ ;  $P = .037$ ) were significantly decreased with 1% w/v MCh. Tissue elastance ( $H$ ;  $P = .151$ ) and hysteresivity ( $\eta$ ) showed no differences compared with the control group.

LPS, lipopolysaccharide; MCh, methacholine.

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after PamCysK4 or LPS in vitro stimulation in LPS-exposed lambs when compared with control lambs (Figure 4, B).

### TGF- $\beta_1$ in lung tissue

At term large amounts of bound TGF- $\beta_1$  were detectable with only a small concentration of free TGF- $\beta_1$  in lung tissue homogenate of the control group (Figure 5, A). In LPS-exposed fetuses, bound TGF- $\beta_1$  in lung homogenate was decreased relative to controls at term ( $P < .002$ ). However, there was no increase in free TGF- $\beta_1$ , which is the biologic active component of TGF- $\beta_1$  that can impair lung development and cause inflammation and airway remodeling. At 7 weeks of age, free TGF- $\beta_1$  concentration was increased in the lung of LPS-exposed lambs ( $P = .048$ , Figure 5, B).

### Elastin

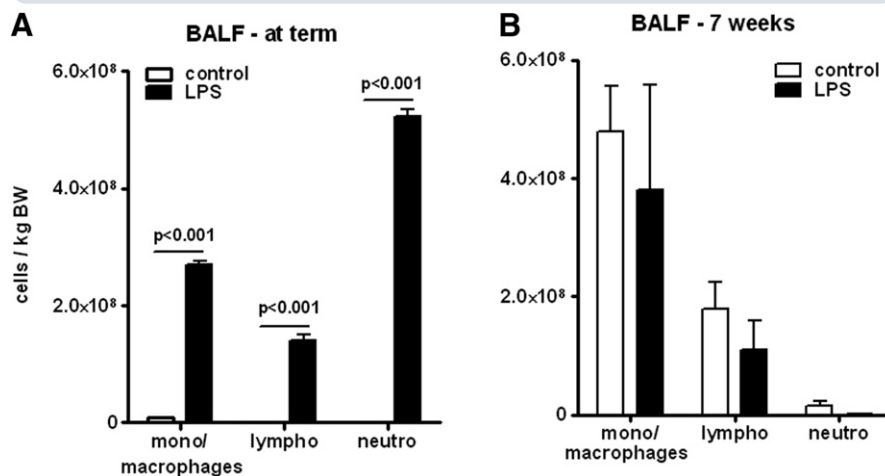
At term, control and LPS-exposed lambs had a normal amount of elastin staining as concentrated elastin foci, with no differences between groups (Figure 6, A) and no differences in the elastin deposition in the blood vessel walls (Figure 6, B and C). At 7 weeks, about 80% of the elastin was nonconcentrated in both animal groups, indicating lungs alveolarization and maturation (Figure 6, D). There was less elastin deposition in the blood vessel walls of the LPS-exposed lungs at 7 weeks of age than in controls (Figure 6, E and F).

### Lymphocytes in the thymus and PMLN

Lymphocyte populations in the thymus and PMLN had an increased percentage of CD4 positive lymphocytes after LPS exposure at term (Figure 7, A). CD8 positive and gamma-delta positive lymphocytes were not different. In contrast, at 7 weeks (Figure 7, B), percentages of CD4 positive were not different in the LPS-exposed lambs compared with the control group. However, in the thymus, there was a lower percentage of CD8 positive lymphocytes and in the PMLN CD8 positive lymphocytes were increased. There was no effect of LPS exposure on gamma-delta positive lymphocytes in thymus and PMLN at 7 weeks of age. Fetal LPS-induced inflammation was associated with an increase in CD4/CD25 double positive lymphocytes

FIGURE 3

Leukocytes in BALF from lambs at term and at 7 weeks of age



Leukocytes in BALF from A, lambs at term and B, at 7 weeks of age. Monocytes, lymphocytes, and neutrophils were increased ( $P < .001$  vs control) in the LPS-exposed group at term. However, no significant differences were evident at 7 weeks, indicating resolution of the pulmonary inflammation present at term.

BALF, bronchoalveolar lavage fluid; LPS, lipopolysaccharide.

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(Figure 7, C) in both thymus and PMLN at term. After 7 weeks, no differences were measured in the thymus. However, the percentage of CD4/CD25 double positive was decreased in PMLN after LPS exposure.

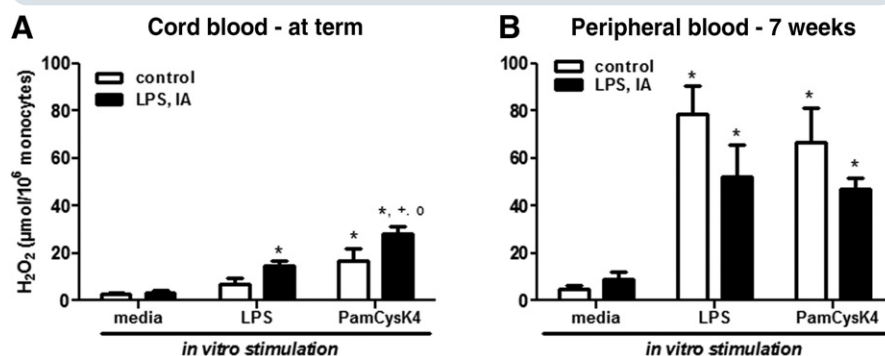
### COMMENT

In this study, we examined the effects of fetal chorioamnionitis induced by 3 IA

injections of LPS from 90 to 110 days' gestation on sheep at term and at 7 weeks of age. In previous experiments was reported profound changes in the pulmonary immune system after fetal exposure to LPS with an increase of immune cells and subsequent maturation of systemic monocytes into PU.1 expressing macrophages.<sup>24,25</sup> In the current study, we also noticed significant increases in pulmo-

FIGURE 4

Hydrogen peroxide production by blood monocytes



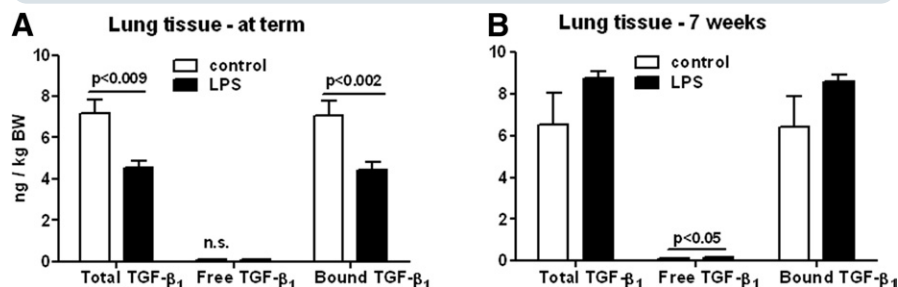
Hydrogen peroxide production by blood monocytes A, at term and B, at 7 weeks of age. At term, hydrogen peroxide production was significantly increased after stimulation of cells with PamCysK4. At 7 weeks of age, this response was reversed, suggesting maturation of the immune system.

LPS, lipopolysaccharide.

\* vs media; + vs control group; (o) LPS vs PamCysK4.

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**FIGURE 5**  
TGF- $\beta_1$  was measured in homogenates of lung tissue from lamb

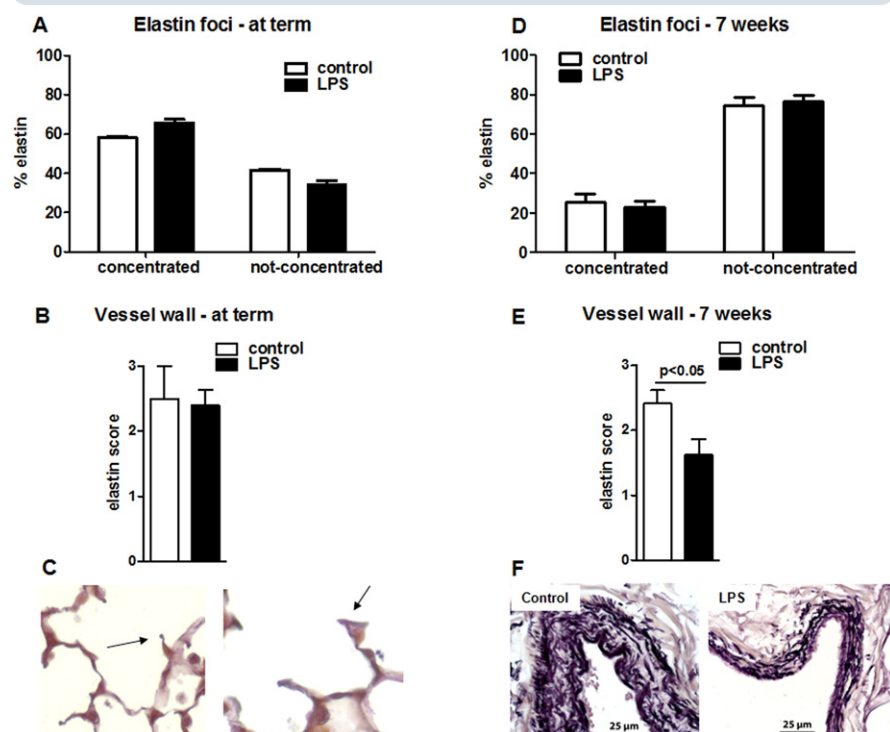


TGF- $\beta_1$  was measured in homogenates of lung tissue from lambs **A**, at term and **B**, after 7 weeks of age. At term, the concentration of total and bound TGF- $\beta_1$  were significantly decreased after LPS exposure, but free TGF- $\beta_1$  was unchanged. At 7 weeks a significant increase of free TGF- $\beta_1$  was detected in the LPS group, but no differences in bound or total TGF- $\beta_1$  were found.

LPS, lipopolysaccharide; n.s., not significant; TGF- $\beta_1$ , transforming growth factor-beta 1.

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**FIGURE 6**  
Elastin in lung tissue from lambs



Elastin (stained in black) in lung tissue from lambs **A**, **C**, at term and **B**, **D**, at 7 weeks of age. **A**, **B**, **E**, Elastin foci were distinguished between concentrated (black arrow) and nonconcentrated (red arrow) foci. **C**, **D**, **F**, Elastin scoring in the vessel wall was performed on a scale from 0-3. At 7 weeks, less elastic fibers were present in the lungs after exposure to LPS. No differences in elastic foci were noticed at term and at 7 weeks of age.

LPS, lipopolysaccharide.

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nary immune cell populations, neutrophils in particular at term. This is remarkable as the fetal LPS exposure was 37 days before the increased neutrophils were detected. Neutrophils were not increased at 7 weeks of age. Monocytes and lymphocytes numbers that were elevated at term remained elevated at 7 weeks of age in lambs exposed as fetuses to LPS. These changes in leukocyte populations may indicate an accelerated development of the pulmonary immune system after LPS-induction.

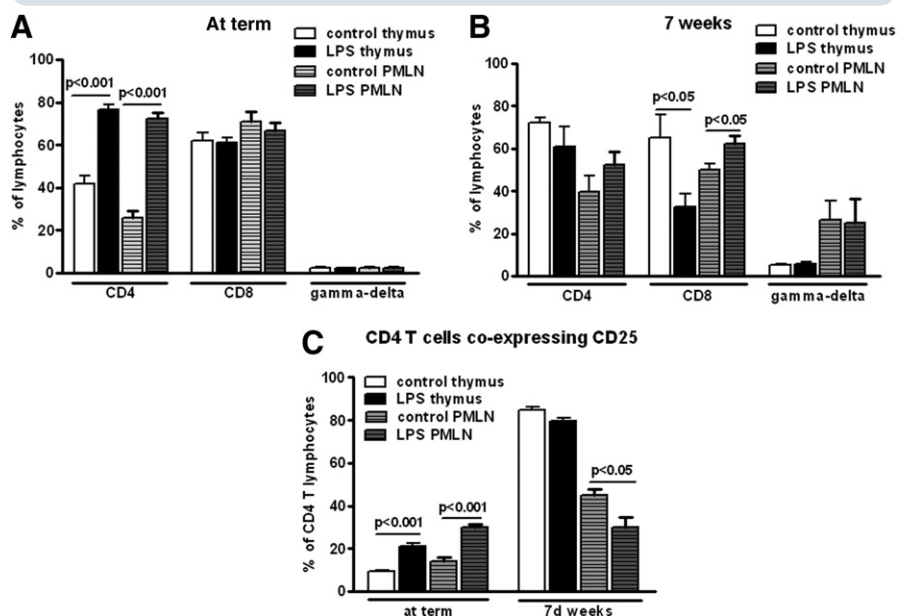
At the systemic level, IA LPS also induced an inflammatory response.<sup>18</sup> Previously, our group showed that the functional response of blood monocytes from preterm LPS-exposed lambs were similar to the blood monocytes of adult sheep.<sup>13,14</sup> A functional assay in the current study demonstrated increased H<sub>2</sub>O<sub>2</sub> production after PamCysK4 (a synthetic ligand for TLRs 1 and 2) stimulation of blood monocytes at term in fetal LPS-exposed animals compared with controls. This result demonstrated that blood monocytes at term were competent to respond to TLR2, because of the fetal LPS exposure. At 7 weeks, controls and LPS-exposed lambs were now similarly able to respond to PamCysK4 stimulation.

Changes in lymphocyte populations of the thymus and the PMLN of LPS-exposed preterm/fetal sheep were demonstrated in previous experiments.<sup>11</sup> Furthermore, LPS-induced chorioamnionitis resulted in a reduced size of the thymus.<sup>23</sup> In the current study, the percentage of cytotoxic lymphocytes decreased in the thymus after fetal LPS exposure, but increased in the PMLN of LPS-exposed lambs. This could be explained by the migration of immune effector cells to the site of inflammation. In addition, the percentage of CD4<sup>+</sup> CD25<sup>+</sup> T lymphocytes were significantly decreased in the PMLN suggesting less suppression of effector cells by regulatory T lymphocytes (Tregs). In the literature, it was proposed that a decrease in Tregs may play a role in the dysregulation of airway inflammation in asthma.<sup>6,26,27</sup> Normally, Tregs can suppress established airway inflammation and airway hyperresponsiveness. Schaub et al<sup>28</sup> demonstrated that cord blood

from offspring of atopic mothers had fewer innate-induced Tregs, indicating a potential mechanism by which intra-uterine immune modulation can occur and may influence further atopic disease development. It also points to the intra-uterine environment as a critical immune modulator. Interestingly, Szépfalusy et al<sup>29</sup> demonstrated that allergens could be transferred across the placenta, supporting the theory that fetal T lymphocytes are exposed during gestation to maternally derived allergens and that the uterus may not be completely sterile. However, the effect of antenatal and postnatal exposure to allergens may have reinforcing effects because exposure to allergens early in life enhanced the development of airway hyperresponsiveness and impairment of lung function at school age.<sup>30</sup>

In our study, we tested the airway responsiveness to metacholine in lambs at 7 weeks of age. Airway resistance and tissue damping did not increase in LPS-exposed lambs at the highest MCh dosage (1.0% MCh) as it did in the controls. Interestingly, these results of decreased airway reactivity are in contrast with clinical observations of increased asthma after chorioamnionitis exposure.<sup>2</sup> However, airway reactivity and immune dysfunction of the lungs do play a role in the development of asthma. The current study demonstrated structural changes in the vascular wall of the lungs. Less elastin was detected in blood vessels but more in lung parenchyma. This finding was surprising because elastin deposition is typically increased in blood vessels in ventilated animal models of lung injury.<sup>31</sup> The low pulmonary blood flow in utero and spontaneous breathing after birth are not comparable to ventilated animal models to our model. Furthermore, decreased concentrations of bound and total TGF- $\beta$ 1 were measured after chorioamnionitis at term. We speculate that the altered deposition of elastin in the vessel wall may be the result of the structural changes initiated before birth and that this is partially mediated by TGF- $\beta$ 1. However, these changes do not explain the decreased airway reactivity. In real life, children are exposed to many allergens, which are additional

**FIGURE 7**  
Percentages of specific T lymphocytes in thymus and PMLN



**A**, At term, the percentages of CD4 lymphocytes were increased significantly in thymus and PMLN (34% and 46%, respectively) of LPS-exposed lambs. After chorioamnionitis, CD8 lymphocytes and gamma-delta lymphocytes were not different. **B**, The percentage of CD8 lymphocytes were decreased by 7 weeks ( $P = .039$ ) in the thymus of the LPS-exposed lambs. However, CD8 lymphocytes were increased ( $P < .028$ ) in PMLN. CD4 and gamma-delta lymphocytes showed no significant changes at 7 weeks. **C**, CD4 coexpressing CD25 lymphocytes were increased in thymus and PMLN of 12% and 16%, respectively, at term. In the thymus no significant changes were present after 7 weeks of age. However, in the PMLN a significant decrease of CD4 lymphocytes coexpressing CD25 was noticed in LPS-exposed lambs at 7 weeks ( $P < .02$ ).

LPS, lipopolysaccharide; PMLN, posterior mediastinal lymph node.

Lee. Fetal effects of chorioamnionitis on 7-week-old sheep. *Am J Obstet Gynecol* 2011.

stimulators for airway reactivity and the later development of asthma. The allergen exposure was not controlled in these free living lambs to 7 weeks of age. Interestingly, Eder et al<sup>32</sup> reported that exposure to LPS in early life protects against allergic sensitization, but not against asthma. This might be an explanation for the different outcome of airway reactivity in our study. Bischof et al<sup>33</sup> demonstrated that age is important for asthma studies in sheep. Significant effects in the airway responsiveness were measured only after 5 months of age. Our studies at 7 weeks may have been in animals that were too young, altered responses to fetal exposure have not been tested previously. Another variable that may change the postnatal outcome is the gestational time of fetal exposure to a proinflammatory stimuli.

In conclusion, our results demonstrate that early fetal exposure to LPS caused residual lung inflammation, but the pulmonary inflammation had resolved by 7 weeks. However, minor structural and functional changes, decreased airway reactivity, and changes in lymphocytes were noted at 7 weeks. The clinical relevance of these results is the demonstration that fetal exposure to chorioamnionitis can modulate postnatal immune status and responses.

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#### REFERENCES

1. Goldenberg RL, Hauth JC, Andrews WW. Intrauterine infection and preterm delivery. *N Engl J Med* 2000;342:1500-7.

2. Kumar R, Yu Y, Story RE, et al. Prematurity, chorioamnionitis, and the development of recurrent wheezing: a prospective birth cohort study. *J Allergy Clin Immunol* 2008;121:878-84.
3. Getahun D, Strickland D, Zeiger RS, et al. Effect of chorioamnionitis on early childhood asthma. *Arch Pediatr Adolesc Med* 2010;164:187-92.
4. Rusconi F, Galassi C, Forastiere F, et al. Maternal complications and procedures in pregnancy and at birth and wheezing phenotypes in children. *Am J Respir Crit Care Med* 2007;175:16-21.
5. Strachan DP. Hay fever, hygiene, and household size. *BMJ* 1989;299:1259-60.
6. Bach JF. The effect of infections on susceptibility to autoimmune and allergic diseases. *N Engl J Med* 2002;347:911-20.
7. Rook G. Review series on helminths, immune modulation and the hygiene hypothesis: the broader implications of the hygiene hypothesis. *Immunology* 2009;126:3.
8. Wolfs TG, Buurman WA, Zoer B, et al. Endotoxin induced chorioamnionitis prevents intestinal development during gestation in fetal sheep. *PLoS One* 2009;4:e5837.
9. Romero R, Mazor M, Wu YK, et al. Infection in the pathogenesis of preterm labor. *Semin Perinatol* 1988;12:262-79.
10. Maloney JE, Adamson TM, Brodecky AV, et al. Diaphragmatic activity and lung liquid flow in the unanesthetized fetal sheep. *J Appl Physiol* 1975;39:423-8.
11. Kramer BW, Kallapur SG, Moss TJ, et al. Modulation of fetal inflammatory response on exposure to lipopolysaccharide by chorioamnion, lung, or gut in sheep. *Am J Obstet Gynecol* 2010;202:77.e1-9.
12. Moss TJ, Knox CL, Kallapur SG, et al. Experimental amniotic fluid infection in sheep: effects of *Ureaplasma parvum* serovars 3 and 6 on preterm or term fetal sheep. *Am J Obstet Gynecol* 2008;198:122.e1-8.
13. Kramer BW, Ikegami M, Moss TJ, et al. Endotoxin-induced chorioamnionitis modulates innate immunity of monocytes in preterm sheep. *Am J Respir Crit Care Med* 2005;171:73-7.
14. Kallapur SG, Jobe AH, Ball MK, et al. Pulmonary and systemic endotoxin tolerance in preterm fetal sheep exposed to chorioamnionitis. *J Immunol* 2007;179:8491-9.
15. Kramer BW, Kallapur SG, Moss TJ, et al. Intra-amniotic LPS modulation of TLR signaling in lung and blood monocytes of fetal sheep. *Innate Immun* 2009;15:101-7.
16. Giannone PJ, Nankervis CA, Richter JM, Schanbacher BL, Reber KM. Prenatal lipopolysaccharide increases postnatal intestinal injury in a rat model of necrotizing enterocolitis. *J Pediatr Gastroenterol Nutr* 2009;48:276-82.
17. Hall GL, Hantos Z, Wildhaber JH, Petak F, Sly PD. Methacholine responsiveness in infants assessed with low frequency forced oscillation and forced expiration techniques. *Thorax* 2001;56:42-7.
18. Kramer BW, Moss TJ, Willet KE, et al. Dose and time response after intraamniotic endotoxin in preterm lambs. *Am J Respir Crit Care Med* 2001;164:982-8.
19. Kunzmann S, Speer CP, Jobe AH, Kramer BW. Antenatal inflammation induced TGF-beta1 but suppressed CTGF in preterm lungs. *Am J Physiol Lung Cell Mol Physiol* 2007;292:L223-31.
20. Kramer BW, Albertine KH, Moss TJ, et al. All-trans retinoic acid and intra-amniotic endotoxin-mediated effects on fetal sheep lung. *Anat Rec (Hoboken)* 2008;291:1271-7.
21. Bland RD, Albertine KH, Pierce RA, Starcher BC, Carlton DP. Impaired alveolar development and abnormal lung elastin in preterm lambs with chronic lung injury: potential benefits of retinol treatment. *Biol Neonate* 2003;84:101-2.
22. Bland RD. Neonatal chronic lung disease in the post-surfactant era. *Biol Neonate* 2005;88:181-91.
23. Kunzmann S, Glogger K, Been JV, et al. Thymic changes after chorioamnionitis induced by intraamniotic lipopolysaccharide in fetal sheep. *Am J Obstet Gynecol* 2010;202:476.e1-9.
24. Kramer BW, Joshi SN, Moss TJ, et al. Endotoxin-induced maturation of monocytes in preterm fetal sheep lung. *Am J Physiol Lung Cell Mol Physiol* 2007;293:L345-53.
25. Cheah FC, Pillow JJ, Kramer BW, et al. Airway inflammatory cell responses to intra-amniotic lipopolysaccharide in a sheep model of chorioamnionitis. *Am J Physiol Lung Cell Mol Physiol* 2009;296:L384-93.
26. Ray A, Khare A, Krishnamoorthy N, Qi Z, Ray P. Regulatory T cells in many flavors control asthma. *Mucosal Immunol* 2010;3:216-29.
27. Robinson DS. Regulatory T cells and asthma. *Clin Exp Allergy* 2009;39:1314-23.
28. Schaub B, Liu J, Hoppler S, et al. Impairment of T-regulatory cells in cord blood of atopic mothers. *J Allergy Clin Immunol* 2008;121:1491-9.
29. Szepefalusi Z, Loibichler C, Pichler J, et al. Direct evidence for transplacental allergen transfer. *Pediatr Res* 2000;48:404-7.
30. Illi S, von Mutius E, Lau S, et al. Perennial allergen sensitisation early in life and chronic asthma in children: a birth cohort study. *Lancet* 2006;368:763-70.
31. Pierce RA, Albertine KH, Starcher BC, et al. Chronic lung injury in preterm lambs: disordered pulmonary elastin deposition. *Am J Physiol* 1997;272(Pt 1):L452-60.
32. Eder W, von Mutius E. Hygiene hypothesis and endotoxin: what is the evidence? *Curr Opin Allergy Clin Immunol* 2004;4:113-7.
33. Bischof RJ, Snibson K, Shaw R, Meeusen EN. Induction of allergic inflammation in the lungs of sensitized sheep after local challenge with house dust mite. *Clin Exp Allergy* 2003;33:367-75.